Research Article

Influence of glucose on cyanidin 3-glucoside absorption in rats

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Anthocyanins are natural dietary pigments that could be involved in various health effects. However their mechanisms of absorption are still not fully understood. The aim of this study was to evaluate the influence of glucose on anthocyanin absorption in rats. We first studied anthocyanin bioavailability in rats that received by gastric intubation ~53 μmol cyanidin 3-glucoside (Cy 3-glc) equivalents from a red orange extract with or without 2.51 mmol glucose. Neither 24-h urinary anthocyanin excretion nor plasma anthocyanin concentration was significantly affected by simultaneous ingestion of glucose. The influence of glucose (12, 42 or 72 mM) on intestinal absorption of Cy 3-glc (pure or from a red orange extract; ~12.3 μM) was further studied using an *in situ* intestinal perfusion model. Absorption of pure Cy 3-glc from the intestinal lumen was not significantly affected by the amount of glucose. However, intestinal absorption of Cy 3-glc from the red orange extract (6.49 ± 1.44%, $n = 6$) was significantly less than that of pure Cy 3-glc (17.5 ± 1.3%, $n = 7$) ($p < 0.01$) suggesting that the red orange extract contained other components that were able to interfere with Cy 3-glc intestinal absorption. This study has thus shown that glucose did not interfere with anthocyanin glucoside absorption.

Keywords: Absorption / Anthocyanins / Cyanidin 3-glucoside / Glucose / Rats

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1 Introduction

Anthocyanins (from the Greek *anthos*, flower, and *kyanos*, blue) are natural dietary pigments widely distributed in fruits and especially in berries [1]. Their average daily intake was estimated for a long time at around 200 mg/day in the United States [2], but much lower intake values were determined by others (6.6 mg/day in Germany and 12.5 mg/day in the United States) [3, 4]. However, intakes higher than 100 mg/day could be easily achieved with regular consumption of red fruits or berries.

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Abbreviations: Cy 3-glc, cyanidin 3-glucoside; **Cy 3,5-diglc,** cyanidin 3,5-diglucoside; **SGLT1,** sodium-dependent glucose transporter 1

Anthocyanins are implicated in many biological activities that may impact positively on health [5, 6]. They may reduce the risk of coronary heart disease, exert anticarcinogenic and neuroprotective activities, reduce inflammatory insult and modulate the immune response [7–9]. These actions might be mediated by their antioxidant activities and/or modulatory actions in cells [10–13].

To better understand the health-enhancing properties of anthocyanins, it is necessary to increase our knowledge of anthocyanin absorption and metabolism. Previous studies have shown that anthocyanins are absorbed at both the gastric and intestinal levels [14–16]. However, the mechanisms of anthocyanin absorption are still not fully understood. It has been previously hypothesised [17–19] that anthocyanin glucosides could interact with the intestinal sodium-dependent glucose transporter 1 (SGLT1), as has been reported for quercetin glucosides [20–22]. However, up to now, there is no evidence suggesting that glucose inhibits anthocyanin absorption by SGLT1.

The aim of this study was thus to evaluate the influence of glucose on the intestinal absorption of anthocyanins (cyanidin 3-glucoside (Cy 3-glc)) using an *in situ* intestinal perfusion model in rats [15]. In addition, we have evaluated urinary excretion in rats that received by gastric intubation a purified anthocyanin extract of red orange containing Cy 3-glc in the presence or absence of glucose.

2 Materials and methods

2.1 Chemicals

Cy 3-glc and cyanidin 3,5-diglucoside (Cy 3,5-diglc) were purchased from Extrasynthèse (Genay, France). The red orange juice extract, free of sugars, was kindly supplied by Professor Francesco Bonina (Faculty of Pharmacy, University of Catania, Italy). Briefly, with the aim to eliminate sugars, fresh red orange juice was filtered twice in a styrene resin column; anthocyanins were then eluted by ethanol/water (60:40). After ethanol evaporation, the extract was lyophilised.

2.2 Feeding study

2.2.1 Animals and diets

Sixteen male Wistar rats born at the Institut National de la Recherche Agronomique and weighing approximately 200 g were housed two *per* cage in temperature-controlled rooms (22°C), with a controlled dark period from 8 to 20 h and access to food from 8 to 16 h. They were fed a semi-purified control diet (755 g wheat starch, 150 g casein, 50 g peanut oil, 35 g AIN-93 M mineral mixture, 10 g AIN-93A vitamin mixture *per* kg) for 10 days [23]. They were then individually housed in metabolic cages fitted with urine and faeces separators and received the control diet for 6 days. The last 2 days of experiment, they received at 8 h by direct stomach intubation either 1.5 mL of a red orange juice extract solution (`Without glucose' rats, $n = 8$) or 1.5 mL of a red orange juice extract solution containing 2.51 mmol glucose (`With glucose' rats, $n = 8$). They have then access to the control diet except for the day of sacrifice. Red orange juice extract was used as a Cy 3-glc source. Administered solutions were obtained by dissolution of the red orange extract at a concentration of 392 mg/mL in water or in 1.67 M glucose solution, respectively. To quantify anthocyanins administered to rats, red orange extract solutions were diluted 3000-fold with 0.12 M HCl before HPLC analysis (20 µL) as described later.

All animals were maintained and handled according to the recommendations of the Institutional Ethic Committee (INRA), in accordance with French decree no. 87-848.

2.2.2 Blood and urine collection

Rats were killed at 1 h after intubation after being anaesthetised with sodium pentobarbital (40 mg/kg body weight). Blood was withdrawn from the abdominal aorta into heparinised tubes and urine present in the bladder was collected. Plasma and urine samples were rapidly acidified with 240 mM HCl. The day before killing, urine was collected over 24 h in tubes containing 1 mL of 3 M HCl. Collection of urine on HCl allowed regeneration of the coloured structure of anthocyanins as urine fell into the tubes and thus increased their stability. All samples were rapidly frozen and stored at −20°C until analysis.

2.3 *In situ* intestinal perfusion

2.3.1 Animals

Thirty-two male Wistar rats weighing approximately 200 g were housed two *per* cage in temperature-controlled rooms (22°C), with a controlled dark period from 20 to 8 h and access to food from 16 to 8 h. They were fed the semipurified control diet for 2 wk.

2.3.2 Anthocyanin administration

Rats fasted for 24 h were anaesthetised with sodium pentobarbital (40 mg/kg body weight) and kept alive under anaesthesia throughout the experiments. After cannulation of the bile duct, a perfusion of the jejuno-ileal segment of the intestine (from 5 cm distal from the duodeno-jejunal flexure up to the ileo-caecal valve) was prepared by installing cannulas at each extremity. This segment was continuously perfused *in situ* for 45 min at a flow rate of 0.75 mL/min with a buffer containing KH_2PO_4 (5 mM), K_2HPO_4 (2.5 mM), $NaHCO_3$ (5 mM), NaCl (50 mM), KCl (40 mM), $CaCl_2$ (2 mM), $MgSO_4$ (1 mM), $K_3C_6H_5O_7$ (10 mM), glutamine (2 mM) and taurocholic acid (1 mM), pH 6.6, at 37°C [15]. The perfused buffer also contained glucose at various concentrations (12, 42 or 72 mM) and pure Cy 3-glc (∼12.3 µM). The 12 mM glucose concentration was considered as a basal one, needed to provide energy to enterocytes and maintain intestinal mucosal integrity. A buffer containing 12 mM glucose and red orange juice extract at a concentration providing the same concentration of Cy 3-glc (∼12.3 µM) was also perfused. The intestine was washed of its contents during the first 25 min. Effluents were directly collected at the exit of the ileum during the last 5 min of perfusion. Effluent volume was estimated by weighing. Bile was collected throughout the 45 min of the experiment. At the end of the experiment, blood samples were withdrawn from the mesenteric vein and abdominal aorta into heparinised tubes. Urine present in the bladder was also collected. Perfused solution, effluent, bile, plasma and urine samples were rapidly acidified with 240 mM HCl and stored at −20°C until analysis.

To determine the stability of Cy 3-glc throughout the *in situ* perfusion experiment (at 37°C, pH 6.6), a sample of the perfused buffer maintained at 37°C was collected at the beginning ($t = 0$), at $t = 25$ min and at the end of the perfusion period ($t = 45$ min), and Cy 3-glc was analysed by

HPLC after acidification with 240 mM HCl, as described later. The overall percentage of degradation was calculated by the decrease in Cy 3-glc concentrations between 0 and 45 min. Anthocyanin degradation was a linear function of time. Thus, the amounts of Cy 3-glc perfused were determined from the mean of Cy 3-glc concentrations in the perfused buffer at $t = 0$ and $t = 45$ min.

2.4 Anthocyanin analysis in biological samples

Urine samples were centrifuged at $12\,000 \times g$ for 5 min and the supernatant fraction (60 μL) was injected and analysed by HPLC as described later.

Anthocyanins present in plasma samples were extracted with a Sep-Pak C_{18} Plus SPE cartridge (Waters, Milford, MA, USA), using Cy 3,5-diglc as internal standard as previously described [14], and then analysed by HPLC (60 μL).

After centrifugation for 8 min at $12\,000 \times g$, the supernatant fractions of intestinal effluents were analysed (20 μL) by HPLC as described later. All the concentrations measured in the effluent samples were corrected by taking into account the intestinal absorption of water. Water absorption was estimated by calculating the difference between effluent flow (estimated by effluent weighting) and perfusion flow (0.75 mL/min). Absorption through the intestinal barrier was estimated by calculating the difference between the amount of Cy 3-glc administered in the intestinal segment and the amount recovered at the end of the ileal segment. These amounts were determined for the last 5 min of perfusion. Anthocyanin stability was also taken into account in evaluating intestinal absorption.

2.5 HPLC analysis

Identification and quantification of anthocyanins were performed by HPLC using a photodiode array detector (DAD 200; Perkin Elmer, Courtabœuf, France) and a UV – Vis detector (785A; Perkin Elmer) at 524 nm. Samples were loaded onto a 150×4.6 mm^2 Uptisphere 3 ODB C18-3 μ column protected by a 10×4 mm^2 Uptisphere 3ODB C18-3 μ guard column (Interchim, Montluçon, France) and analysed as previously described [23]. Peak identification and assignment were based on the comparison of their retention time and spectral data with that of red orange extract, standards or our previous data obtained by HPLC-MS/MS analysis [17, 24]. Anthocyanin quantification was expressed as Cy 3-glc equivalents.

2.6 Data analysis

Values are given as means with their standard errors. When appropriate, significance of differences between mean values was determined by one-way analysis of variance followed by a Student – Newman – Keuls test (GraphPad; Instat, San Diego, CA). Values of $p < 0.05$ were considered significant.

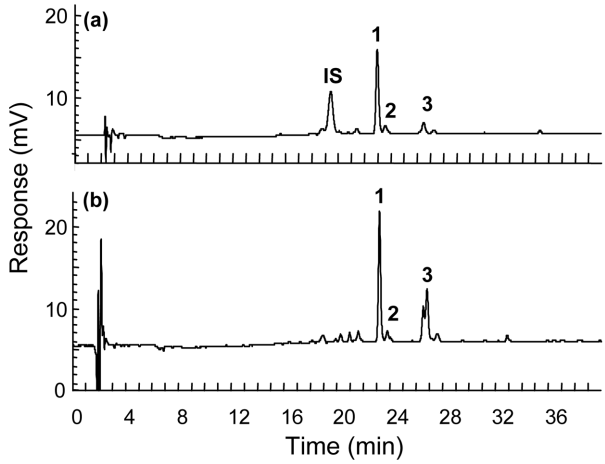

Figure 1. HPLC chromatograms of (a) plasma and (b) 24-h urine from rats receiving 1.5 mL of a red orange extract solution. Detection was performed at 524 nm. Peaks are as follows: IS internal standard Cy 3,5-diglc, (1) Cy 3-glc, (2) cyanidin monoglucuronide, (3) methylated Cy 3-glcs + methylated cyanidin monoglucuronide.

3 Results

3.1 Feeding study

Rats received 1.5 mL of red orange anthocyanin solution, *i.e.* ~53 μmol Cy 3-glc equivalents with or without 2.51 mmol glucose. The HPLC profiles of urine and plasma showed Cy 3-glc as well as methylated and/or glucuronidated derivatives (Fig. 1). Urine collected directly in the bladder and 24-h urine both presented the same HPLC profile.

Neither 24-h urinary anthocyanin excretion nor plasma anthocyanin concentration ($t = 1$ h) were significantly affected by simultaneous administration of glucose (Table 1). Around 0.13% of the anthocyanins ingested were recovered in 24-h urine as native Cy 3-glc and metabolites. Cy 3-glc represented, respectively ~44 and ~65% of total urinary and plasma anthocyanins.

3.2 *In situ* intestinal perfusion

The overall percentage of Cy 3-glc degradation (pure or from red orange extract) during the 45 min of perfusion was less than 5%. The HPLC profile of effluents was similar to that of the perfused solutions. Absorption of pure Cy 3-glc from the intestinal lumen was not significantly affected by the amount of glucose in the perfused solution (Table 2). However, intestinal absorption of Cy 3-glc from red orange

Table 1. Urinary anthocyanin excretion and plasma anthocyanin concentration following oral administration of red orange anthocyanins with or without glucose[a, b)]

Rats	Anthocyanin ingestion (μmol/24 h)	Urinary anthocyanin excretion		Plasma total anthocyanin concentration (nmol/L)	Plasma Cy 3-glc[c)] concentration (nmol/L)
		(nmol/24 h)	(% of the ingested amount)		
`Without glucose'	52.3	64.7 ± 6.2	0.124 ± 0.012	250 ±16	164 ± 10
`With glucose'	52.9	71.4 ± 9.9	0.135 ± 0.019	225 ±22	147 ± 18

Results are expressed as Cy 3-glc equivalents.
a) Rats received by direct stomach intubation 1.5 mL of a red orange juice extract solution contaning or not 2.51 mmol glucose. Plasma anthocyanin concentration was determined 1 h after stomach intubation.
b) Mean values with their standard errors ($n = 8$).
c) Cy 3-glc, cyanidin 3-glucoside.

Table 2. Anthocyanin absorption after perfusion of Cy 3-glc with various concentrations of glucose through the intestinal lumen of rats[a)]

Compounds[b)]	Cy 3-glc perfused through the intestinal lumen[c)] (nmol)	Cy 3-glc absorption from the intestinal lumen[c)]	
		(nmol)	(% of the perfused dose)
Cy 3-glc + 12 mM glu	49.6 ± 5.4	8.81 ± 1.27	17.5 ± 1.3
Cy 3-glc + 42 mM glu	52.0 ± 6.6	7.06 ± 1.26	13.3 ± 0.9
Cy 3-glc + 72 mM glu	43.3 ± 6.1	6.11 ± 0.98	13.6 ± 1.3
Red orange + 12 mM glu	50.0 ± 10.2	2.96 ± 0.61*	6.49 ± 1.44*

* $p < 0.01$ *versus* ‚Cy 3-glc + 12 mM glu'.
a) Mean values with their standard errors ($n = 6-8$).
b) Cy 3-glc, cyanidin 3-glucoside; glu, glucose.
c) These amounts correspond to the last 5 min of perfusion.

extract was significantly less than that of pure Cy 3-glc ($p < 0.01$). Cy 3-glc as well as its methylated derivative was detected in urine collected after *in situ* intestinal perfusion. No anthocyanin was detected in plasma or bile samples owing to the low amount of anthocyanins perfused.

4 Discussion

The aim of the present study was to evaluate the influence of glucose on anthocyanin absorption in rats. Indeed, although some studies have hypothesised that anthocyanin glucosides could interact with the intestinal SGLT1 [17–19], available data are scarce.

The ingestion of 30 g sucrose by healthy volunteers has been shown to result in a slightly reduced urinary excretion of elderberry anthocyanins [19]. Moreover, Bub *et al.* [18] reported that mean time to obtain maximal plasma concentration of malvidin 3-glucoside was delayed after ingestion of red grape juice as compared to red wine or dealcoholised red wine in humans. They have thus concluded in favour of a time delaying effect of the sugar present in red grape juice which could result from a competitive action of glucose and malvidin 3-glucoside on SGLT1 as reported for quercetin 3-glucoside [20, 21]. An opposite result was reported by Frank *et al.* [25] since the rate and extent of anthocyanin glucoside absorption was enhanced following drinking of red grape juice compared with red wine. However, sugar content was not the unique difference between these drinks and it was thus difficult to unequivocally conclude on the influence of glucose.

In the present study, we have used a red orange juice extract deprived of sugar as Cy 3-glc source and administered it to rats with or without 2.51 mmol glucose. Expressed as dry weight, rats eat approximately 20 g/day diet whereas humans eat in the region of 400 g/day food. Therefore, the consumption of 2.51 mmol (*i.e.* 450 mg) glucose by rats would correspond to about 9 g in human subjects, *i.e.* the amount contained in about 300 mL red orange juice. Rats were sacrificed 1 h after the last anthocyanin administration, *i.e.* when plasma anthocyanin concentration was close to its maximum [26, 27]. At this time, plasma anthocyanin concentration was not affected by glucose. Moreover, our study showed that simultaneous ingestion of glucose and anthocyanins did not affect 24-h urinary anthocyanin excretion (~0.13% of the ingested amount) that was of the same order of magnitude as previously reported (for review, see [28]). Thus our results indicate that anthocyanin bioavailability was not influenced by glucose administration in the rat.

This study also evaluated intestinal absorption of pure Cy 3-glc in the presence of various amounts of glucose

using an *in situ* intestinal perfusion model [15]. The 12 mM glucose concentration in the perfused buffer is a usual and physiological amount in such experiments [15, 29]. Since we have previously observed that intestinal absorption of Cy 3-glc from a red orange juice solution containing a higher amount of glucose (72 mM) was lower than that of pure Cy 3-glc perfused with only 12 mM glucose [15, 17], we have tested in the present study this high amount and a middle one (42 mM). The amount of glucose in the perfused solution did not affect the intestinal absorption of pure Cy 3-glc. This result is in accordance with a recent *in vitro* study [30] that has shown using mouse jejunum mounted in Ussing chambers that D-glucose (up to 40 mM) had no significant effect on Cy 3-glc (5 μM) disappearance from the mucosal solution. Both studies thus suggest that SGLT1 is not involved in anthocyanin absorption. Moreover, in the presence of 12 mM glucose, the intestinal absorption of Cy 3-glc from the red orange extract was dramatically lower than that of pure Cy 3-glc. This last result, which is in line with our previous work [17], suggested that the red orange extract contained other components that were able to interfere with Cy 3-glc intestinal absorption. In this respect, it is worth noting that Walton *et al.* [30] have recently shown that the flavonol quercetin 3-glucoside inhibits Cy 3-glc absorption *in vitro*. However, we have controlled that the red orange extract did not contain significant amounts of flavonols and flavanones (data not shown). Simultaneous ingestion of other components could thus affect anthocyanin absorption. It would be thus important to take this point into consideration since anthocyanins are rarely ingested on their own.

In conclusion, the present study showed that contrary to what was described for flavonols [20 – 22], glucose did not affect anthocyanin glucoside absorption. However, the influence of other flavonoids or food matrix on anthocyanin absorption needs further investigation.

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The authors have declared no conflict of interest.

5 References

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